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# Laboratory Culture of the Sepiolid Squid *Euprymna scolopes:* A Model System for Bacteria–Animal Symbiosis

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**Abstract.** The small Hawaiian sepiolid *Euprymna scolopes*, with its symbiotic luminous bacterium *Vibrio fischeri*, was cultured through one complete life cycle in 4 months. Paralarval squid hatchlings were actively planktonic for the first 20–30 days, after which they settled and assumed the typical adult mode of nocturnal activity and diurnal quiescence. Squids were aggressive predators that preferred actively swimming prey up to 2–4 times their size; the only diet that yielded good survival and rapid growth for paralarvae was large adult mysids. Survival to settlement was 73% on this diet, whereas it was 0%–17% on controls and three other diets. Paralarvae initially lacked both detectable luminescence and *V. fischeri* cells in their incipient light organs; all remaining stages produced luminescence, and their light organs were colonized by apparently pure cultures of $>10^5$ *V. fischeri* typical of *E. scolopes* symbiont strains. Survival from settlement to sexual maturity was 76%. Mating and egg laying commenced at 2 months, yet attempts to culture the next laboratory generation of hatchlings were not as successful. The results indicate that the host organism of this symbiosis can soon be cultured with consistency through its brief life cycle, thus opening new avenues of research into developmental aspects of this symbiosis.

## Introduction

The symbiotic association between the Hawaiian sepiolid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* has been developed into an experimental marine model in cell and molecular biology (Wei and Young, 1989; McFall-Ngai and Ruby, 1991; Ruby and McFall-Ngai, 1992). Various strains of *Vibrio fischeri* (both natural and mutant) have been cultured so that components of the symbiotic association can be manipulated experimentally (*e.g.*, Boettcher and Ruby, 1990; Ruby and Asato, 1993; Graf *et al.*, 1993; Dunlap *et al.*, 1995). However, full development of this marine model system has been hampered by the inability to culture the host organism—the squid—completely through its life cycle with some degree of consistency and standard methodology.

*Euprymna scolopes* is a very small species that is endemic to the Hawaiian Islands, where it spends much of its life buried in the sand. There have been no field studies of its behavior and life cycle, so most of what is known comes from laboratory studies (Singley, 1983). Of particular interest is how these small squids might use their relatively huge light organ in their daily lives.

Since the mid-1980s, several teams of researchers have studied details of the symbiosis by bringing wild-caught adults to the laboratory, allowing them to mate and lay eggs, and then using the hatchling squids (*i.e.*, the paralarvae) to explore the infection process as well as the initial development of the light organ. However, thus far investigators have been unable to examine events beyond the first week posthatching because the squid paralarvae deplete their internal yolk supplies by that time and perish; several unreported rearing attempts in the 1990s have failed. Nevertheless, before this model system was developed, *Euprymna* had been reared from

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eggs through a portion of its life cycle. *Euprymna berryi* was reared to 2 months in Korea by Choe and Oshima (1963) and Choe (1966). Arnold *et al.* (1972) reared 10 *E. scolopes* (from a starting number of 26) to 28 days, and two survived to 202 days without reproducing. Singley (1983) reared 13 of 16 *E. scolopes* to 28 days and one to 120 days, although its reported length was very small (<4 mm mantle length). The encouraging results of Arnold *et al.* (1972) and Singley (1983) on *E. scolopes*, coupled with the rapid development of this symbiosis model system in the last 5 years, led us to initiate the trials reported here. The overall goal was to develop standard methods for culturing *Euprymna* and to learn aspects of its biology that would enhance its successful culture in captivity. We hypothesized that behavioral features related to feeding would be most important to the successful culture of *Euprymna* hatchlings through the paralarval stage, as they have proved to be in many other squid species (*e.g.*, Boletzky and Hanlon, 1983; Yang *et al.*, 1986; Hanlon *et al.*, 1989; Hanlon, 1990; Lee *et al.*, 1994).

Although we hope to eventually culture successive generations of this small, fast-growing cephalopod, a near-term goal is to develop a method for rearing aposymbiotic hatchlings (*i.e.*, those deprived of the bacterium) through most of the life cycle, so that cellular and molecular aspects of the complex development of the symbiosis can be studied in even greater detail. We report here our initial findings that *Euprymna scolopes* can be cultured through its brief life cycle under controlled laboratory conditions, and we highlight some of the more important needs of this species in captivity. We also describe our observations on some critical features of the behavior of this species, including multiple effects of light and some aspects of reproductive biology that require future experimentation.

## Materials and Methods

The term "paralarvae" is used to describe young squids from hatching to about 3 weeks of age; it accurately distinguishes the behavior of hatchlings from that of juveniles and adults (see details in Young and Harman, 1988). *Euprymna scolopes* is a member of the family Sepiolidae in the order Sepioidea (according to the nomenclature of Voss, 1977), which includes cuttlefish. The term "squid" is commonly applied to some sepioids, although true squids are members of the order Teuthoidea.

### *Brood stock acquisition and egg care*

Adults were collected on Oahu, Hawaii, and shipped via airfreight to the Marine Resources Center of the Marine Biological Laboratory in Woods Hole, Massachusetts. Squids were shipped individually in 3-l plastic bags containing 1.5 l of natural seawater. The seawater was filtered to 10 $\mu$m, heavily aerated, and spiked with 1 g/l tris buffer; the remaining 1.5 l of each bag was filled with oxygen and the bags were fitted in insulated shipping boxes. The total time spent in these bags by each squid was typically 21 hours. When the bags arrived at the laboratory, the temperature was slightly depressed (down to 18°–19°C), the salinity was 31–32 ppt, the concentration of dissolved oxygen was 7–11 mg/l, the pH was 8.29–8.41, and the levels of ammonia and nitrite were low (however, colorimetric reading were often impossible due to interference from ink in the bags). Ammonia was measured with LaMotte kits that are based on the salicylate method and colorimetric analysis. Nitrite and nitrate were measured with Hach kits that also used colorimetric methods; the precision of the ammonia and nitrite methods is ±0.01 mg/l, and the lowest sensitivity is 0.05 mg/l.

Adult males were housed individually, and females individually or in pairs, in small chambers (25 cm × 33 cm; water depth 18 cm) and were fed *ad libitum* with live shrimp (*Palaemonetes* sp. or *Crangon* sp.). Each chamber had 10 cm of crushed coral sand for the squids to bury in.

Mating was induced once per week by transferring one male into a female's chamber overnight. To avoid damaging the squid they were transported in small clear glass vials of water rather than in nets. Transferred males always mated, and eggs were generally laid the night after the male was removed. In the trials reported here, five females and three males produced 13 clutches of eggs, but we used only five clutches for the culture trials.

It was essential that eggs be handled as little as possible so that they would develop fully and not hatch prematurely. The key was to keep temperature, salinity, pH, nitrogen levels, and light cycles as steady as possible, and to keep disturbance at a minimum (not bumping tanks, changing light levels, inspecting eggs, *etc.*). Adults were kept in closed, recirculating seawater systems to ensure that water quality was consistent. Eggs laid on coral fingers were left on the coral and moved to aerated mesh containers. Eggs laid on the flat tank wall were gently scraped from the wall with a glass slide; they generally came off as a single unit, which was then transferred to a mesh container. Eggs were never exposed to air, and an airstone was placed into each mesh container adjacent to the eggs so that water was constantly circulating near them. The eggs were kept in the same water that adults had laid them in.

### *Rearing chambers and seawater systems*

The actual rearing chamber for each paralarval rearing trial was a circular black container, 25 cm in diameter,

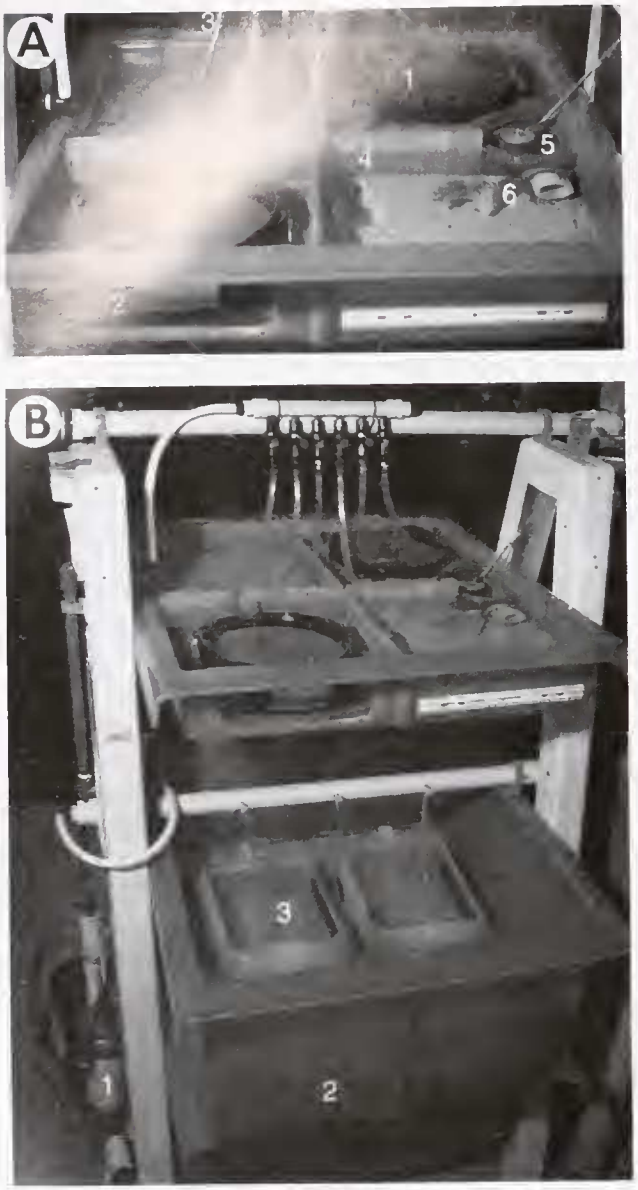


Figure 1. The culture tanks for *Euprymna scolopes*. (A) Each paralarval rearing chamber (1) held 30 squids and had a viewing port (2). Water flowed in through two tubes (3) in each chamber and exited vertically through the sand-covered sieve bottom, then flowed horizontally through mesh screens (4) to the drain (5). The white ruler is the standard 12 inches. (B) The complete closed system and A-frame, showing the filter apparatus (1), the location of the biological filter substrate (2) and the chambers for maintaining adults and for mating (3).

with a 200-$\mu$m-mesh screen bottom (Fig. 1). The chamber was immersed in a seawater tray tank to achieve shallow water depths of only 3–6 cm. Water flowed into the top through rubber tubing and flowed out through the mesh bottom. The two inflow tubes were arranged along the edge to create a gentle circular water flow, which helped keep the hatchlings and prey items away from the sides. A thin layer of sand coated the mesh bottom.

Two small seawater systems were used in the Marine Resources Center: one was a completely recirculating system of 340 l, and the other was an open system of 600 l. Local Woods Hole water was the original source, and this water was passed through a 1-$\mu$m filter and heated to about 23°C. The closed system consisted of an A-frame with a shallow tray tank for the rearing experiments above (70 cm × 78 cm × 9 cm deep) and a deeper tank below to house the biological filter. The biological filter was composed of crushed oyster shell with an undergravel filter; the oyster shell was 15 cm deep and spread over an area of 5250 $cm^2$. The water was then pumped through a canister with a particulate filter and then through activated carbon and a UV filter. The flow rate was about 22 l/min, and about 30% of the water was replaced weekly. The open system was constructed similarly, with a top tray size of 76 cm × 130 cm × 9 cm deep and a tank below with 9956 $cm^2$ crushed oyster shell as a substitute substrate. The open system was only used for some squids after Day 49 to help reduce crowding. Immersion heaters in each system helped maintain temperature. Water quality was checked two or three times per week and ammonia, nitrite, and nitrate were determined according to the methods listed above.

The tanks were situated 3 m from a window that provided indirect natural light. Overhead fluorescent fixtures provided indirect light on a 12:12 light cycle. The typical light level falling on the tray tanks was 2–8 $W \cdot m^{-2}$ $\mu$A.

One small trial ($n$ = 8 squids) was performed in a semi-closed seawater system at the Marine Science Center of Northeastern University. Natural seawater was used in an A-frame that held a total of 350 l. Crushed coral was the biological filter, and no other UV or charcoal system was used. Lighting was by direct overhead incandescent bulbs (50 cm away) on a 12:12 cycle. The paralarvae were reared in a black circular PVC chamber (20 cm in diameter, 20 cm in height) with 150-$\mu$m-mesh sides and a bare plastic bottom.

For behavioral observations, the top trays were fitted with glass panels for horizontal viewing into portions of the round rearing chambers. Observations were made from the side and also from the top of the tanks, and night observations were accomplished with a night-vision device that amplified existing dim light. A very weak red light was aimed at the ceiling to produce a soft glow of reflected light in the room (approximately 0.3 W $m^{-1}$ $\mu$A). A video camera (Sony HiBand 8 mm) was used to record behavior; the night-vision device could be fit to the front of the camcorder to record nocturnal behavior.

*Hatchlings, stocking densities, and foods*

Most embryos hatched in the first 2 h of the dark cycle, and individuals were transferred with a turkey baster to the culture chamber. For individual 30-day feeding trials, 30 hatchlings were stocked in each round rearing chamber. The exceptions were one trial in which 50 hatchlings were stocked and one trial in which only 8 hatchlings were taken to the Nahant laboratory. By day 50, squids were removed from the small round chambers and the juveniles were reared in the divided tray tanks, each of which had dimensions of 35 by 39 cm.

Food items included live zooplankton, crustaceans, and larval fishes. Mysid shrimps of the genera *Mysidopsis* and *Neomysis*, and larval fishes, *Menidia beryllina*, were commonly used. The term "postlarval mysids" was applied to those that were newly hatched from the brood sacks of females (Lussier *et al.*, 1988). They were of body lengths 0.5–1.5 mm, whereas adult mysids were typically 4–10 mm long. Hatchlings were fed 1–5 times per day between 0700 and 2300 to ensure adequate food availability. Progressively larger shrimp (*Crangon* and *Palaemonetes*) were provided to juvenile and adult squids as they grew.

*Luminescence measurement and quantification of symbiotic bacteria*

Light produced by hatchlings, juveniles, and subadults was detected qualitatively with a Turner 20e luminometer (Sunnyvale, CA). Individual live animals were placed in 5 ml (50 ml for subadult animal) of filter-sterilized (0.2-$\mu$m pore size) natural seawater in 30-ml glass scintillation vials (300-ml glass beaker for subadult animal, placed in a foil-lined funnel to channel light into the luminometer detector), and the light produced was recorded for 30 s. Three measurements were taken. The data reported (as arbitrary light units, LU, per animal) are the highest of the light levels detected for each animal.

*V. fischeri* cells colonizing the animal light organ were quantified by plate counts using a seawater complete agar (SWC; Nealson, 1978). To minimize the presence of surface-associated bacteria, hatchlings and juveniles were removed from culture tanks in a minimal volume of seawater and rinsed by three passages in 5 ml of filter-sterilized seawater in autoclave-sterilized scintillation vials. The animals were then homogenized in a 15-ml Ten Broeck tissue homogenizer with 1 ml of filter-sterilized seawater. The homogenate was serially diluted and plated in quadruplicate on SWC agar plates, which were incubated at room temperature for 24 h before colonies were counted. For subadults, the light organ was removed aseptically and homogenized and handled as above. Representative colonies of bacteria arising on the spread-plates, which were uniform in appearance for those animals that produced light, were confirmed to be *V. fischeri* strains that characteristically colonize light organs of *E. scolopes* (Boettcher and Ruby, 1990; Dunlap *et al.*, 1995) by (i) their production of luminescence, although at a very low level in culture, (ii) their luminescence response to the *V. fischeri* autoinducer-producing, nonluminous strain MJ-203, and (iii) their effect on the luminescence of the autoinducer nonproducing strain MJ-215 (Kuo *et al.*, 1994; Kuo *et al.*, 1996).

## Results

*Hatching success, water quality, and system design*

An essential improvement that led to successful culture was providing a very stable environment so that embryos developed to full term and did not hatch prematurely. Failure to do this affected early trials adversely. Temperature, light, and pH were nearly constant, and the nitrogen levels were kept within standard limits (*i.e.*, 0.10 mg/l for ammonia and nitrite, 20 mg/l nitrate; Spotte, 1973; Hanlon, 1990). Hatching occurred after about 18–22 days at 21–23°C. Particular care was taken not to bump the tank or handle the eggs during the last days of development. A sample check under a dissection microscope indicated that hatchlings had no external yolk amidst the arms, and normal amounts of internal yolk along the midgut (Arnold *et al.*, 1972).

Concentrations of ammonia ($NH_3$) and nitrite ($NO_2$) remained near the recommended levels of 0.10 mg/l except for two brief spikes of ammonia to 0.40 mg/l on day 22 and 0.20 mg/l on day 71, and one brief spike of nitrite to 0.20 mg/l on day 46. No mortalities or unusual behavior could be correlated with these spikes. Nitrate ($NO_3$) levels were very low throughout the trials, ranging from 1 to 4 mg/l, well below the recommended level of 20 mg/l. The pH always stayed above 8.0, and salinity ranged from 30 to 37 ppt although the mean was near 35 ppt. Temperature ranged from 21 to 25°C, but on one occasion the temperature in the Northeastern University tank decreased slowly to 4°C during the night. The temperatures were returned to 21°C in the course of 5 h. All of these tropical Hawaiian squids survived!

The utility of a shallow rearing chamber was evident; in particular it helped concentrate prey organisms for the very young squids. The system also allowed easy visual inspection of the tank, including the behavior of the squids and their food sources.

*Behavior of paralarvae and juveniles*

The paralarvae in this study showed two notable behavioral features: (i) they were strong swimmers, especially compared to other cephalopod hatchlings of com-

parable size; and (ii) they were alternately active and planktonic as well as benthic for the first month post-hatching. The first feature was validated in field observations when RTH observed the hatchlings and juveniles in their natural habitat in Hawaii in August 1995. Very small squids were able to dart towards prey 20 cm away in only a second or so, a large distance for a paralarval cephalopod.

Diurnal paralarval swimming activity is illustrated in Figure 2. There were many squids swimming during the day in both the unsuccessful and successful feeding trials. Nevertheless, even the youngest hatchlings spent a large proportion of their day buried in the sand substrate. By day 30 in our trials, all of the squids remained benthic during the day like the adults. At all phases of the life cycle, the squids were active throughout the night (only occasional observations were made from midnight to 0600 h). The behavior of juveniles was seemingly identical to that of adults: they remained buried in the sand during the day and hunted for prey at night.

At all life stages, *Euprymna* tolerated high densities. By the time the squids had settled (*ca.* 30 days) and grown for a total of 40–50 days, their density was quite high considering that they were still in the small round rearing chambers. At this time some were transferred to an adjacent tank system, and in both systems the squids were distributed into rectangular sections measuring 35 × 39 cm with a sand substrate of about 1–2 cm. There were no cases of cannibalism, which is common in many cephalopods.

Unlike the squid in most of the trials, the group of eight taken to Northeastern University were given no sand substrate. These squids never sat on the bottom but swam all the time. After 2–3 weeks, they settled on the bare PVC bottom; the five squids that survived in this system were brought to Woods Hole and reared with others to adulthood.

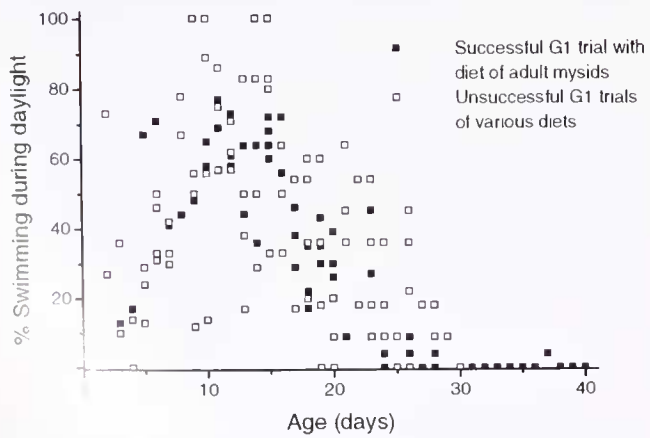


**Figure 2.** Paralarval swimming behavior, indicating the percentage of squids that was actively swimming during daylight hours. After 30 days all squids remained benthic during the day.

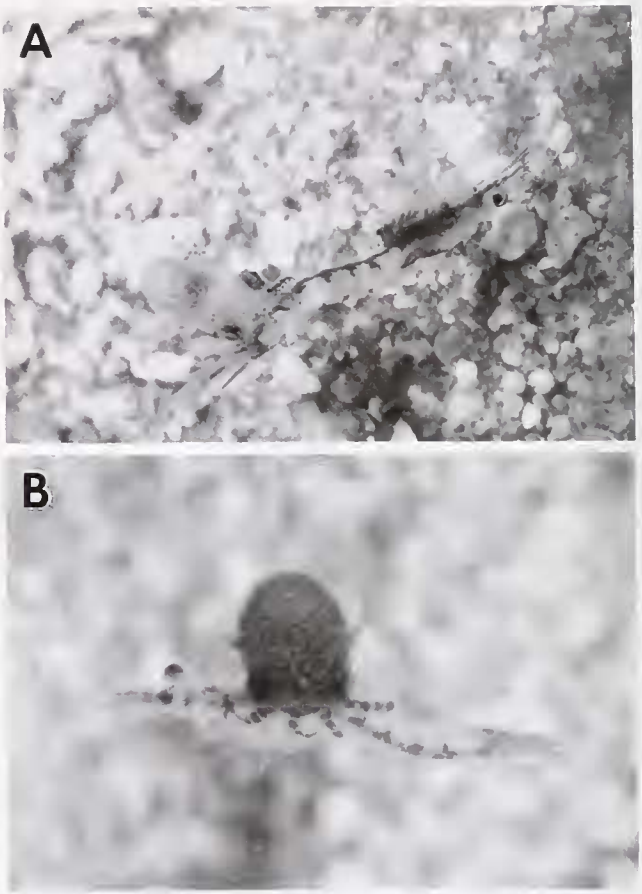


**Figure 3.** Predation by paralarval squids. (A) A 15-day-old paralarva subduing a very large mysid, which it ate successfully. (B) A 7-day-old paralarva eating a mysid and holding it in a typical position at mid-carapace.

*Feeding*

Substantial effort was devoted to studying the feeding behavior of squids, the swimming behavior of various prey, and the interactions of predator and prey. The most important finding was that hatchlings of *Euprymna scolopes* are voracious predators that prefer very large prey relative to their own size. As indicated in Figure 3, it was common for hatchlings to successfully attack and ingest prey that were much larger than themselves. Conversely, they were capable of eating very small prey, on the order of 0.5 mm. Hatchlings were already strong swimmers and could easily make vigorous forward attacks of 12 body lengths in 1 s (as measured from videotape). Field observations at night in Hawaii also confirmed this strong swimming ability.

To assess different preferences, six diets were tried for

the critical paralarval period of 1–30 days post-hatching. Figure 4 indicates the clear results: large adult mysids were preferred and also produced the best survival; 22 of 30 squids survived to settlement. These mysids were usually stocked at levels of about 2–4 per squid, but the numbers fluctuated a great deal. Control animals received no food and all died by day 8, as did all 30 squids provided with a smorgasbord of live zooplankton. Postlarval mysids, which were expected to be the best food, produced poor results too; only 5 of 50 squids survived to settlement. When postlarval mysids and larval fishes were provided together, the mortality was initially high but stabilized within a week; 5 of 30 squids survived to settlement. Similar results were obtained with irregular mixed diets that consisted of postlarval mysids and fishes, as well as zooplankton and adult mysids; only 4 of 30 survived to settlement. It is worth emphasizing that food was presented in abundance in every case, and for all but a few of the 30 days in each trial.

The behavior of squids during active periods (*i.e.*, when in the water column) seemed to be related solely to foraging. Very rarely was a prey item attacked near the substrate. Generally the squids preferred to strike upward, which often required that they descend under the prey, then move rapidly towards it at about a 45 degree vertical angle. Because their two tentacles were not evident until about 25 days post-hatching, squids used their eight arms to grasp the prey. Feeding was clearly stimulated by the addition of prey items to the tank; possibly the increased motion and swimming activity of prey was enough of a stimulus to provoke more attack attempts by squids. For this reason it is advisable to feed squids many times per day rather than once or a few times. Mysids were clearly preferred, even when other prey such as fish larvae were present in the tank in far greater quantities. Squids were commonly observed to make 2–8 strikes at mysids before a successful capture. Usually a large mysid was held at mid-carapace (Fig. 3B), and it is possible that the squids were biting through the dorsal nerve cord to immobilize the mysid. We occasionally saw two squids eating the same large mysid and, more rarely, one squid attacking a mysid while in the process of eating another; this occurred only with the smaller postlarval mysids. Extremely large mysids (*e.g.*, more than 4 times the size of squids) were not eaten and probably disrupted the squids in small chambers.

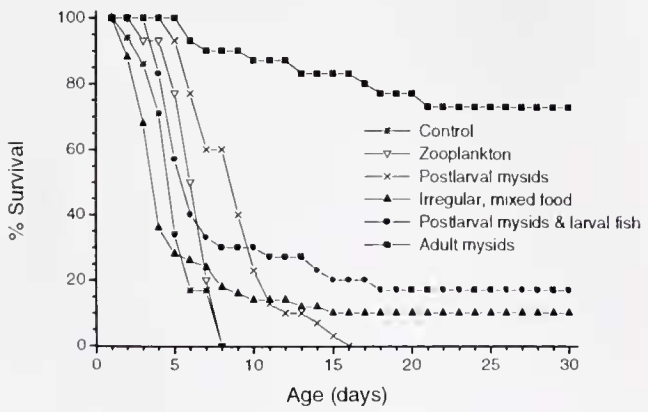


**Figure 4.** Survival on different diets during the 1-month paralarval period.

Paralarval squids showed considerable interest in fish larvae (*Menidia*) on some days. But the *Menidia* larvae seemed to present problems for capture, and survival of squids on this diet was poor. For example, on day 27, squids were presented with fishes that were nearly 12 mm long. The squids strongly pursued the fish larvae, yet often made 4–6 unsuccessful strikes and sometimes as many as 20; recall that squids of this age and size were well developed and strong swimmers. In a typical feeding, only 1–3 squids would have a fish 15 min after 30–40 fishes were added and 30 attacks were observed. By contrast, if 30 shrimp (*Crangon*) were placed in the same tank, commonly 8 squids would have successfully captured one within 5 min. It was often observed that squids could not hold onto a fish even when they made contact, suggesting that the suckers were not able to grasp the fish well. Once captured, fish were harder to subdue than shrimp; it took on the order of 2–3 min, as compared with 10–30 s for shrimp. When a fish was being eaten, the stomach of the squid was black and highly visible compared to a much less distinct dark color when shrimp were ingested.

Food densities varied greatly due to variable food supply. However, in the early weeks, about 2–4 large adult mysids were supplied to each squid in trial 1. These mysids, which were about 1–3 times the average body length of the very young squids, were generally eaten in the course of 24 h. By days 35–40, these squids were being fed a combination of adult mysids and *Crangon* shrimp (1.0–1.5 cm length) at a level of 5–6 times as many prey as predator; the tanks appeared very crowded when the food was first put in. By day 52, 15 juvenile squids were being fed about 20 *Crangon* (1.5 cm length) and 100 large mysids.

Paralarval squids foraged throughout day and night, but this was not quantified by counting the number of attacks on prey per unit time. After settling to the adult behavior mode by day 30, all squids foraged at night, and food was generally added to tanks late in the day and cleaned out the following morning. Juveniles and adults continued to feed vigorously on prey that were generally their own size or larger.

Lighting levels appeared to be important to successful feeding: lower light enhanced feeding, whereas bright light seemed to retard it. For example, when tops were

placed over the rearing chamber during the day, the reduced light level stimulated feeding. Very dark overcast days also resulted in [illegible] squids spending more time foraging and [illegible] light in the rearing chambers was arrange[illegible] individual squids could view prey objects in [illegible] ding light against a black background, and this [illegible] increased the contrast of the prey organism.

*Growth, age, maturity, and mortality*

Growth was rapid and adult sizes were reached in about 2 months. Figure 5 illustrates growth in mantle length and wet weight over the culture period. Hatchlings ranged from 1.6 to 1.9 mm in mantle length and from 4.2 to 5.8 mg in wet weight. Despite the limited data set (*i.e.*, few points between days 10 and 70) and the highly conservative growth measurements (nearly all taken from freshly dead squids), rapid growth in wet weight through day 83 was still best fit by the exponential equation $y = 0.0033e^{(0.084x)}$. From this function, the instantaneous relative growth rate from hatching to day 83 was an 8.4% increase in weight per day. After day 83, the data were not amenable to curve fitting because growth was very slow and the data were highly variable. The extremely high growth rate of paralarvae is mostly due to the high feeding rate; hatchlings that were feeding on 2–3 very large mysids per day were probably ingesting more than their body weight per day. The length/weight relationship, based upon 41 measurements from hatching to day 133, was expressed by the equation

Wet weight in grams

$$= 0.0015 \times (\text{mantle length in millimeters})^{2.674}$$

The complete life cycle (*i.e.*, from egg to egg) was completed in about 80 days, and the longest-lived squid reached an age of 139 days. As noted in the next section, many squids were sexually mature by 60 days post-hatching. During the paralarval stage, mortality was 27%, then the population stabilized and the remaining mortalities occurred over a protracted period that was marked by reproductive activity. From settling to sexual maturity, mortality was only 24%; thereafter, there was a slow attrition. Early mortalities were probably related to nutrition, but later ones were inexplicable and may have been associated with maturity and "old age" or with some unknown pathogen.

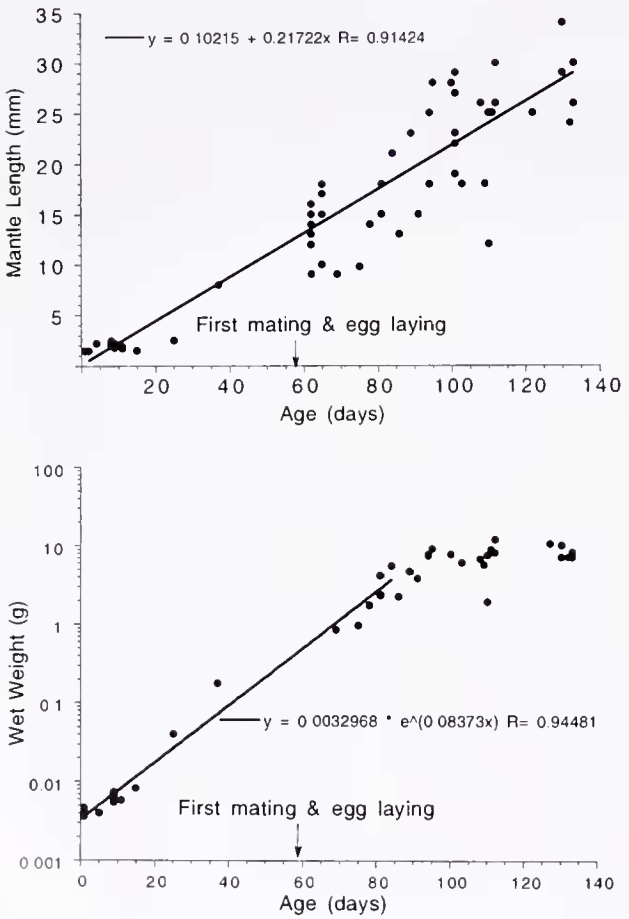


Figure 5. Growth of cultured *Euprymna scolopes*. TOP: Linear plot of mant e length increases versus age. BOTTOM: Wet weight growth was exponential through day 83 ($R = 0.95$). Data for males and females are lumped because identification was not always possible.

*Reproduction*

Sexual dimorphism is only slightly evident in this species. Males have slightly enlarged suckers on some arms and tend to have slimmer posterior mantles, especially compared to fully mature females whose posterior mantles become broader as they fill with eggs. The testis of the male can sometimes be seen dorsally when all the chromatophores are retracted.

The first eggs were laid on day 58 and the first mating was observed on day 61, when 24 squids were still alive. Mating occurred at night, often just at the onset of darkness. Overall, 16 matings were observed between days 61 and 116; the last squid died on day 139, so that mating occurred over the last one-third to one-half of the brief life cycle. Matings were not controlled in any way and possibly more matings occurred throughout the nights than we observed. Mating (Fig. 6A) seemed to be initiated by the male (bottom), who grasped the female and placed a spermatophore somewhere in her mantle. Mating lasted about 30–50 min in the few cases in which the whole mating was observed. Competition for mates was observed only once: two males grabbed a female, some wrestling followed, then they all moved to corners of the tray tank. One male was removed, but the remaining

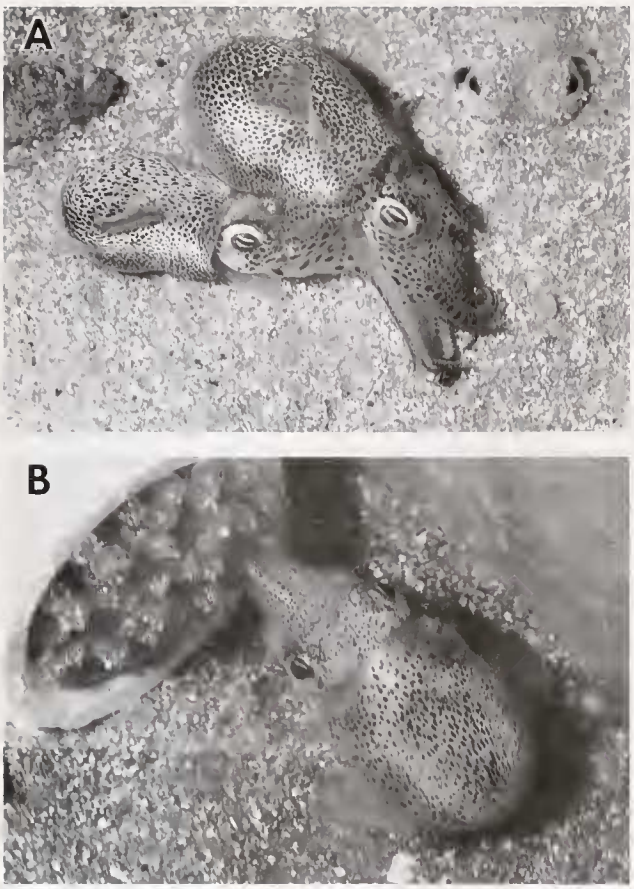


**Figure 6.** Mating (A) and egg laying (B) by cultured *Euprymna scolopes*. The male grasped the female from underneath and they mated for 30–50 min. Later, the female affixed one egg at a time to a PVC pipe and coated the eggs with sand to camouflage them.

male and female did not mate that night. In this particular case, the female was larger than the males.

Egg laying (Fig. 6B) was observed four times and, curiously, took place in the mornings and often lasted until midday. In total, 13 egg clutches were laid by this generation of adults. Attachment of each egg took 10 s on average, and about 40 s elapsed between the deposition of each egg, so that it took about 25–30 min to lay a clutch of 30 eggs. As usual, the eggs were soon coated with sand that was somehow placed there by the females.

To minimize handling, individual squid were not marked; thus fecundity could be estimated only from the number of eggs laid by individual females. All tray tanks contained males and females in high densities, even when the squids were separated on day 49; *i.e.*, there were 2–3 squids per 35 × 39 cm chamber.

*Reestablishment of the bacterial symbiosis in reared squids*

To determine whether symbiosis with *V. fischeri* was reestablished in the light organ of the reared squids, hatchlings, juveniles, and subadult animals were examined for the production of light and for the presence of *V. fischeri* cells. The hatchling squids initially lacked luminescence, and no colony-forming units of *V. fischeri* were detected in their nascent light organs. By day 5, however, the animals were luminous, and their light organs contained $10^5$ or more *V. fischeri* cells (Table I). Figure 7 illustrates bacterial cells in the light organ of a reared squid.

## Discussion

We have identified three essential keys for successful laboratory culture of *Euprymna scolopes:* (i) the eggs must be provided with conditions that lead to complete embryonic development, thus rendering fully competent hatchlings; (ii) water quality must be good, and the tank configuration and lighting must be tailored to the specific needs and behavior of the species; and (iii) the proper type and quantity of prey organism must be provided. Observations in the sea as well as in the laboratory indicated that *E. scolopes* was a voracious predator for its size, and that relatively large prey were preferred.

Because our main interest in culturing *E. scolopes* is to advance the use of this model of symbiosis, it was also important to demonstrate that laboratory animals were competent to receive bacteria in a manner similar to that of wild-caught *Euprymna*. Our results are consistent with previous demonstrations that hatchlings initially are aposymbiotic and that they acquire symbiotic strains of *V. fischeri*, and consequently produce light, within about a day of hatching (Wei and Young, 1989; McFall-Ngai and Ruby, 1991). Furthermore, squids that had reached the juvenile benthic and subadult stages also produced luminescence, and their light organs contained increasingly larger populations of *V. fischeri* (Table I; Fig. 7).

**Table I**

*Re-establishment of bacterial symbiosis in cultured* Euprymna scolopes

| Animal stage | Approximate size (mm) | Animal light production[1] | Symbiont CFU[2] |
|---|---|---|---|
| Hatchling (Day 0–1) (Planktonic) | 2 × 2 | not detected ($n$ = 5) | not detected ($n$ = 5) |
| Paralarval (Day 5) (Planktonic) | 2.5 × 2 | 44<br>3.2 | $6.0 \times 10^5$<br>$5.0 \times 10^5$ |
| Juvenile (Day 15) (Benthic) | 4 × 3 | 220 | $8.0 \times 10^6$ |
| Juvenile (Day 30) (Benthic) | 7 × 4.5 | 16 | $2.0 \times 10^7$ |
| Subadult (Day 130) | 35 × 20 | 640 | $8.0 \times 10^7$ |

[1] LU (arbitrary light units) per animal.
[2] Symbiont CFU = colony-forming units of *Vibrio fischeri*

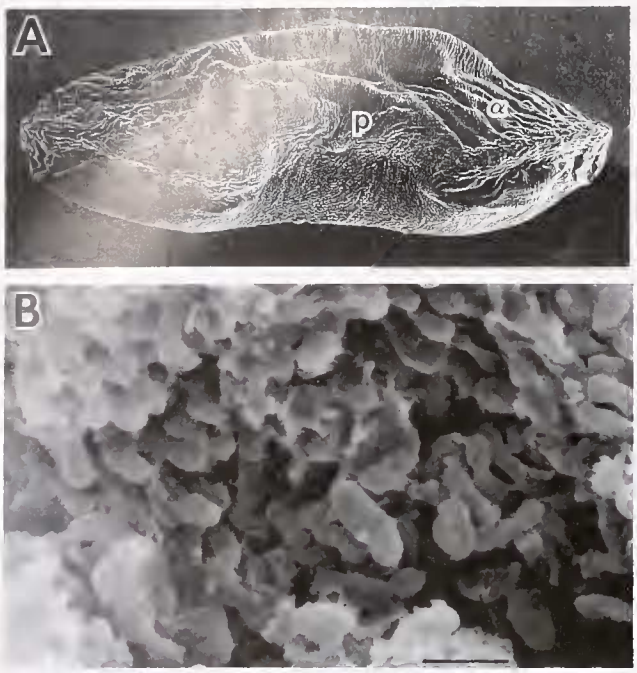


**Figure 7.** (A) Scanning electron micrograph of half the light organ of a mature, cultured squid. Letter "p" indicates the pore. Symbol indicates the approximate region of interest shown in panel B. (B) High magnification of *Vibrio fischeri* cells within the internal cavity of the distal end of the light organ. Bar is 1 μm.

*Behavior, life cycle, and laboratory culture comparisons*

It has been found repeatedly that teuthoid and sepioid squids are visual predators that generally prefer actively swimming prey (*e.g.*, Boletzky *et al.*, 1971; Boletzky and Boletzky, 1973; Boletzky, 1974; Boletzky and Hanlon, 1983; Yang *et al.*, 1986; Hanlon *et al.*, 1989; Hanlon, 1990; Lee *et al.*, 1994). The peculiar decapod arrangement of eight arms and two tentacles allows capture and ingestion of both very small and unusually large prey. Nevertheless, as observed in many of the studies just cited, many seemingly good prey items are not preferred by paralarval squids, thus diets must be determined experimentally. It was predictable that *E. scolopes* would ingest mysids given the enormous success of this diet for other squids (studies cited above) and cuttlefish (Forsythe *et al.*, 1994) and the results of rearing work on *E. berryi* (Choe and Oshima, 1963; Choe, 1966) and *E. scolopes* (Arnold *et al.*, 1972; Singley, 1983). It was *not* predictable that *E. scolopes* paralarvae would prefer such large prey and only survive well on them as compared to mysids of other sizes. However, field observations made with a night-vision device by RTH in Hawaii indicated that very young *E. scolopes* were exceptionally strong swimmers that could jet forward at great lengths. Laboratory video in this experiment documented forward attacks of at least 12 body lengths in about a second, a feat that small squids such as *Loligo* cannot perform.

How might the paralarvae live in nature? Our study indicates that their activity patterns are flexible compared with those of adults (Fig. 2). They can bury in the sand like adults, yet they are surprisingly strong swimmers that can capture prey with a very rapid forward attack and avoid predators by combining a swift backwards jet escape with inking. We did not see any evidence that the light organ was used by paralarvae, juveniles, or adults in our study, despite many observations at night. It seems unlikely that paralarvae commonly use the light organ while seeking prey because we probably would have observed it through the viewing ports in the side of the tray tanks (Fig. 1). A more plausible use for the bioluminescence would be in defense against predators from below, when *Euprymna* of any life stage are higher in the water column. Since the organ is directed downward, it probably is used to eliminate or interrupt the squid's shadow against the downwelling light.

*Euprymna scolopes* has one of the shortest life cycles of any cephalopod (Boyle, 1983), mainly as a result of its rapid exponential growth, the warm temperatures it lives in, and its small adult size. The fast exponential growth through day 83 is typical for many squids; since first mating and egg laying were seen at about day 60, we expected that growth would slow substantially soon thereafter, which it did. The shortest life cycle reported for a cephalopod is in the small tropical species *Idiosepius pygmaeus:* statolith ring analysis indicates that this species matures in 1.5–2.0 months and lives only about 79 days (Jackson, 1989); this species was not cultured in the laboratory. *E. scolopes* is relatively easy to rear because of the large strong hatchlings and their propensity for mysid shrimps. Close relatives of *E. scolopes* share rapid growth, small size, and preference for mysids. *Euprymna berryi* was reared to 2 months by Choe and Oshima (1963) and Choe (1966); four species of *Sepiola* and two of *Sepietta* were reared by similar techniques by Boletzky *et al.* (1971); *Sepietta oweniana* was cultured by Summers and Bergstrom (1981); and *Rossia macrosoma* was reared to 8 months by Boletzky and Boletzky (1973). Only *E. scolopes,* however, lives in warm water, which accelerates growth (Forsythe and Van Heukelem, 1987) and promotes a short life cycle. Could these techniques be applicable to *Euprymna morsei* from Japan and to similar sepioids with light organs?

*Behavioral and physiological factors that require future attention*

Now that progress has been achieved with the paralarval stages, the next logical stage is to focus on reproductive behavior. Practically nothing is known about the mating system of any member of the subfamily Sepioli-

nae (*e.g.*, Boletzky *et al.*, 1971; Boletzky, 1975; Moynihan, 1983; also reviewed by Hanlon and Messenger, 1996), and optimal conditions for brood stock management will have to be determined before "normal" reproductive behavior and high fecundity can be expected. The density of adults, the diet, the light cycle (especially gradual changes that imitate natural changes), the combinations of females and males and the nature of their pairings, agonistic behavior, courtship, and any form of sperm competition will all influence mating, egg laying, and the quality of the progeny. The matings observed in this study averaged 35 min; Moynihan (1983) and Singley (1983) reported matings of 25–80 min. These long mating times combined with the presence of a seminal receptacle (called the pharetra, located internally near the opening of the oviduct) strongly suggest the possibility of sperm competition behavior among males. A practical problem is to determine how many adult squids must be maintained as brood stock under optimal conditions (physical and social) to ensure sufficient genetic diversity in subsequent generations.

A misbalance in the mating system can put stress on the females, resulting in poor egg production, low fertilization rates, and thus poor hatching rates or lack of vigor in the progeny. Crowded laboratory conditions in cuttlefish and loliginid squids can lead to forced copulations of females (J.G. Boal, pers. comm., 1996) and a disruption of the mating system. Lack of attention to these key issues in reproduction is one reason why most cephalopods cultured in captivity have not been cultured through multiple generations. Another sepioid, the cuttlefish *Sepia officinalis*, is the one cephalopod that has successfully been cultured through many generations (Forsythe *et al.*, 1994), and is currently in the 14th laboratory generation at the University of Texas Medical Branch in Galveston.

The second-generation hatchlings in this culture trial did not do well, but no obvious cause was detected. Because we had no access to a control group of hatchlings from wild-caught adults, we were unable to determine whether the problem lay with our techniques or with a lack of vigor in the first filial generation. We also had no certain clues to the cause of the mortalities that occurred throughout the trials, although several of the adults had whitish patches of ulceration that are common in many laboratory-reared cephalopods (Hanlon and Forsythe, 1990). Certainly other factors should be analyzed more closely—for example, different light cycles and types of substrates (*cf.*, Shears, 1988)—to see how they affect the health and well being of squid in captivity.

### *Future possibilities for this marine model of symbiosis*

The immediate application of our culture techniques is in exploring new questions about the developmental biology of this symbiosis. The light organ requires bacteria before it can develop (Montgomery and McFall-Ngai, 1994), and the bacteria need the light organ to become luminescent (Boettcher and Ruby, 1990). On one hand, the ability to culture the host organism—the squid—opens the prospect of studying late development of the light organ, although it first requires that the paralarvae be reared in the absence of the bacteria. On the other hand, one can now address issues of involvement of bacteria in developmental programs at the level of tissue, organ, and whole animal.

The longer-term application of *Euprymna scolopes* culture is to develop this species into a genetic model. This is not a trivial task, but *E. scolopes* has characteristics that favor success. Unlike other cephalopods, this species is small and short-lived, and each female usually lays its clutch of eggs in a single night and in a discrete clutch, so that parentage and reproductive success can be assessed.

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